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PREVALENCE OF ANTIBODIES TO *COXIELLA BURNETII* IN BULK MILK SAMPLES IN ENGLAND AND WALES

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Un test ELISA a été mis au point pour détecter les IgG anti C. burnetii dans des échantillons de lait de mélange. La spécificité du test a été validée par absorption croisée avec C. burnetii, C. psittaci et des antigènes de l'œuf. Un échantillon aléatoire de 373 fermes laitières sur 19.000 a été réalisé. Du petit lait de ces fermes a été étudié initialement à une dilution standard contre un sérum bovin positif et deux échantillons de lactosérum positif ont été mélangés et utilisés comme référence. Les échantillons restants ont été alors testés de nouveau et leurs titres comparés avec cette référence. En l'absence d'un véritable échantillon de lactosérum négatif, il a été difficile de déterminer la valeur seuil. Ces résultats sont discutés en relation avec la constatation que 11 échantillons (2,9 p. cent) étaient positifs par rapport au lactosérum de référence.

INTRODUCTION

Q fever is a zoonosis caused by the rickettsial organism *Coxiella burnetii*. Each year, approximately 100 human Q fever infections are reported in England and Wales (Pebody et al. 1996). Recent work has demonstrated an association between contact with cattle and human infection with *C.burnetii*; calving cattle and handling the products of conception gave relative risks of 1.63 and 1.45 respectively (Thomas et al., 1995). The current *C. burnetii* status of the UK national cattle herd is unknown. The most recent estimates, from the 1950's, suggested a prevalence in cattle herds of 0.8% in Scotland, 2.0% in Wales and 6.9% in England (Slavin, 1952). The use of bulk milk testing of cows' milk for the presence of antibodies to a variety of pathogens provides a convenient way of sampling a whole herd (Emanuelson et al., 1989) and has been used successfully to identify herds infected with BVDV (Niskanen, 1993). This study describes the development of an enzyme-linked immunosorbent assay (ELISA) to detect antibodies to *C.burnetii* in bulk milk samples of dairy cattle and its' use to estimate the proportion of herds with antibodies in England and Wales.

MATERIALS AND METHODS

Sample collection

The largest bulk milk collection company in the United Kingdom handles approximately 75-80% of the milk produced in England and Wales. Samples from each farm are taken daily, or every two days, in bar-coded pots and transported to the company's milk quality testing laboratory within 24 hours. A list of random numbers, generated on the company's mainframe computer, was used to obtain a random selection of bulk milk samples. A sample size of 377 was required to estimate the proportion of herds with antibodies to *C.burnetii* in this population of 19,000 milk producers, assuming a 50% infection rate with 95% confidence and ±5% precision. Whey was separated from all samples by centrifugation at 44,000G for 2 hours at 4°C. Separated whey was stored at -20°C.

Standard sera

Bovine serum samples, with titres greater than 1/40 in a complement fixation test (CFT) for antibody to *C.burnetii*, were used as positive controls to develop the ELISA. Negative controls were serum samples obtained from 6-24 month old, high security housed, SPF-derived cattle. No known positive or negative milk samples were available.

Indirect enzyme-linked immunosorbent assay (ELISA)

The ELISA used whole cell, egg-derived, formalin-killed, phase II *C.burnetii* antigen. The wells of flexible PVC microwell plates were coated with 100µl of antigen at a 1:1,200 dilution (approx. 1µg/well) in sodium carbonate coating buffer (pH 9.7) and incubated at 4°C for 24 hours. The plates were then washed 6 times in phosphate buffer (pH 7.4) with 0.1% Tween (PBST) and shaken dry. Wells were then blocked with 200µl of 1:15 rabbit serum in coating buffer at 4°C for 24 hours. Following washing as before, 100µl dilutions of samples and standards in PBST were incubated at 4°C for 4 hours. Four coated wells were incubated with PBST only to record plate background. Plates were again washed before 100µl of affinity-purified rabbit-anti-bovine, whole molecule, IgG alkaline phosphatase conjugate (Sigma Co., UK) at 1:5,000 in PBST was added to each well and incubated at 4°C for 2 hours. The plates were given a final wash and dry before 100µl of substrate 104® (Sigma Co.,UK) was added at 1:10,000 in coating buffer. Plates were left to develop at room temperature before optical densities (OD) were read at 405nm once the positive standard exceeded 0.5. During the development of the test several different plate types, blocking sera and proteins, incubation times

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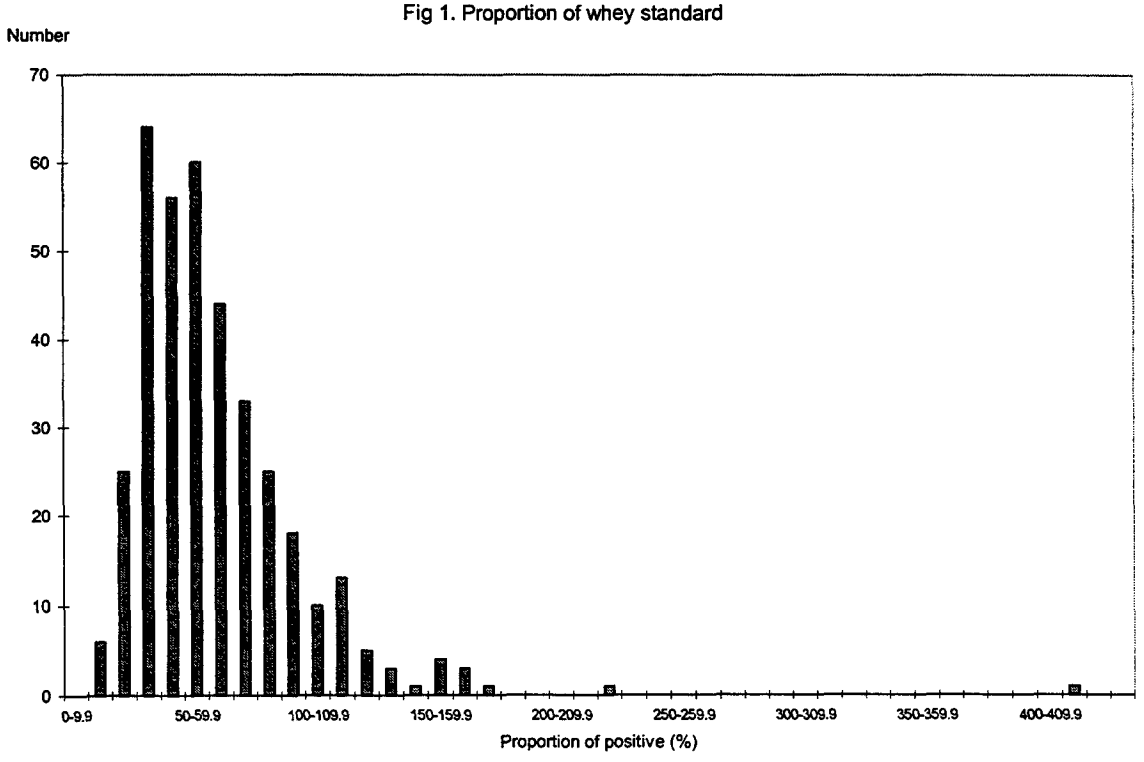
and temperatures were tried in an attempt to reduce non-specific binding of serum and whey. The immunological specificity of the ELISA was tested by absorption of antibody from positive sera. Aliquots of positive serum were incubated overnight at 4°C with *C.burnetii* grown on yolk sac membrane, *Chlamydia psittaci* cultured in a similar manner and uninfected yolk sac membrane. The aliquots were then centrifuged and used as test sera in the ELISA.

Screening of whey samples for *C.burnetii* antibody and estimation of their antibody titres
The whey samples were initially screened in duplicate, at a 1:300 dilution, against a 1/160 CFT positive serum diluted at 1:3,000, 1:9,000 and 1:27,000. Each plate also included a negative serum at the same dilutions as the positive serum. Two strongly positive whey samples were identified from the results of the screen. These two whey samples were pooled and used as a positive standard. To estimate the titre of antibody, six doubling dilutions of this positive whey standard (starting at 1:30) were compared with four doubling dilutions of each sample (starting at 1:20). A further two samples from the initial screen were pooled and run on each plate to confirm the reproducibility of the ELISA.

Data analysis
In order to identify positives in the preliminary whey screen, the average OD of the whey samples was examined graphically and displayed as a proportion of the 1:3000 positive standard. The maximum dilution of the positive serum was used as the cut-off point. Whey titres were calculated by comparing the samples with the positive whey standard using the Elisanalysis 5.01 programme (Dr Peterman, Birmingham, USA). Distributions and descriptive statistics were calculated using Minitab 10 (Minitab Inc., USA).

RESULTS
Four samples were lost in transit, therefore whey was obtained from 373 (98.9%) bulk milk samples.
Incubation of positive serum with the *C.burnetii* antigen reduced the OD to background, but the OD was not reduced by incubation with either uninfected yolk sac membrane or *C.psittaci*.
268 samples had OD readings above twice the plate background. When values obtained from a 1:27,000 dilution of the positive serum standard were used as a cut-off point, 32 (8.6%) milk whey samples appeared to contain antibody to *C.burnetii*. When the final titres of whey antibody were compared with the standard pooled milk whey they ranged from 14.0 to 410.8% and were log-normally distributed (Fig.1).
Removal of a single outlier provided the best estimate of the Anderson-Darling Normality Test (p= 0.942) for the logarithmically transformed distribution of these titres. The 95% upper confidence interval for this distribution was at 143.2% of the positive whey standard. Eleven (2.9%) samples lay above this point and were considered to be positive.

Fig 1. Proportion of whey standard



DISCUSSION

An ELISA test was developed to detect antibody to *C.burnetii* in bulk milk samples. The immunological specificity of this test was established by absorption with *C.burnetii*, the medium in which it was grown and a related intracellular organism. Since *C.burnetii* is an airborne pathogen, one of the major problems encountered in this study was the absence of a negative whey sample. Negative serum samples were obtained from animals housed in high security units. Furthermore, although parallel dilution curves were obtained for positive serum and spiked whey (data not shown), the lack of a known positive whey is also a source of concern because the concentration and subtype population of antibodies is different between serum and whey.

Randomly selected bulk milk samples were used to gain an unbiased estimate of infected dairy herds in England and Wales. However, the use of Milk Marque for the collection of samples will in itself have produced some selection bias since the company collects milk from only 70-75% of these herds.

The whey screening was performed at the dilution which provided the greatest differentiation between positive and negative serum sample. The estimate of 8.6% of dairy herds in England and Wales with antibodies to *C.burnetii* is similar to estimates of infection obtained by Slavin (1952) In the absence of whey samples from a population of known unexposed cows, the cut-off point remains a major problem with this study. We have attempted to address this problem using the techniques suggested by Vizard et al. (1990). By choosing a cut-off point using a log-normal distribution of the data, an estimate of 2.9% of infected herds was obtained.

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